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ANALYSIS OF ASSOCIATION BETWEEN TLR-4 ASP299GLY AND THR399ILE GENE POLYMORPHYSIMS AND CHRONIC PERIODONTIS IN BABYLON POPULATION, IRAQ

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ABSTRACT

To analyze the association between toll like receptors-4 asparginine299glycine and therionine399ilucine gene polymorphisms and chronic periodontitis**.**Genomic DNA was obtained from peripheral blood of 40 patients with chronic periodontitis and 30 periodontally healthy controls. TLR-4 Asp299Gly and Thr399Ile gene polymorphisms were genotyped by a polymerase chain reaction-restriction fragment length polymorphism method. The data were analyzed by calculation of Odds ratio (OR) with 95% confidence interval (95%CI) using logistic regression analysis. Statistical significance was set at a p value≤ 0.05.Theallele's frequency of Thr399Ile gene was higher among CP group (82.5%) than control (63.3%) with significant difference. The prevalence of a Thr399Ile heterozygote was found to be 10% in the chronic periodontitis group and zero in the periodontally healthy group.There was significant association between receptor-4 therionine399ilucine and chronic periodontitisand no significant difference in the toll like receptor-4 asparginine299glycinegene polymorphism between the chronic periodontitis and controls.

KEYWORDS: Chronic Periodontitis, TLR-4 Gene, Polymorphisms

INTRODUCTION

Periodontitis is a chronic inflammatory disease of complex etiology. Although the presence of microbes especially *P.gingivalis* G-ve anaerobes is essential in initiating and perpetuating periodontitis, environmental as well as genetic factors contribute to individual variation in the susceptibility to disease [5].

Periodontal destruction is a multistep process consisting of microbial infection, host immune response, and tissue destruction. Risk factors can affect any of these stages through genetic and environmental interactions. The innate immune system is implicated in the recognition of conserved pathogen associated with molecular patterns present on the pathogens [1] and recognition of these molecules is facilitated by a group of receptors called Toll-Like Receptors (TLRs). Among the 10 human TLRs identified so far, TLR-2 and TLR-4 are the most defined members [10, 21]. TLR-4 has been shown to specifically identify the lipopolysaccharide (LPS) of G-ve bacteria which effort in cooperation with several protein components such as LPS-binding protein (LBP) and CD14 and this leads to activation of inflammatory cells via the nuclear factor-Kappa beta pathway (NF-KB) which results in the synthesis and release of proinflammatory cytokines, there by augmenting the local inflammatory response [22, 3].

Arbour et al. [4] found two common gene polymorphisms of the TLR-4 are ASP299Gly and Thr399Ile polymorphisms which had been shown to be associated with functional changes that predispose people to be less responsive to LPS, attenuate the TLR signaling pathway and leading to a dull inflammatory response thus have an increased risk of susceptibility to G-ve infections.

Several studies by Schröder et al. [19] and Brett et al. [6] had described the role of TLR-4 gene polymorphism in patients with periodontitis and have found association between chronic periodontitis and TLR-4 gene polymorphism. As frequency of polymorphisms and gene environment interactions differ among populations. Hence the aim of this study is to evaluate association between the TLR-4 Asp299Gly and Thr399Ile gene polymorphisms and chronic periodontitis in Babylon population. The objectives of this study were to determine the genotype distributions and alleles frequencies of TLR-4 SNP among CP patients and controls in Babylon population and to estimate the risk of chronic periodontitis among the different genotypes of TLR-4.

MATERIALS AND METHODS

The study involved a total of 70 individuals who reported to Department of Periodontics were included in this study. The subjects were divided into a chronic periodontitis group (40) and a control (30). The chronic periodontitis group included 23 males and 17 females ranging in age from 40 years to 65years, exhibiting clinical attachment loss ≥5 mm. The control group participants (17 males and 13 females) ranging in age from 30 years to 55 years were included. Smokers, subjects with a history of cardiovascular disorders, diabetes, malignant disease, immunodeficiency, and subjects with a previous history of periodontal surgery were excluded. Informed consent was obtained from all subjects.

Genomic DNA Preparation

Five milliliters of venous blood was collected by vein punctures and placed in a 15 ml sterile centrifuge tube containing ethylenediaminetetraacetic acid (EDTA). Human genomic DNA was isolated from the leukocytes using the modified Miller's 1998 protocol .

PCR- RFLP

Determination of the TLR-4 gene polymorphism was accomplished with PCR and restriction fragment length polymorphism by the method of Lorenz et al. [15].

STATISTICAL ANALYSIS

The Statistical Package for the Social sciences version 18 (SPSS Inc., Chicago, USA) was used for statistical analysis. The association between genotype and risk of CP was estimated by calculation of Odds ratio (OR) with 95% confidence interval (95%CI) using logistic regression analysis. Statistical significance was set at a p value≤ 0.05.

RESULTS

The allele and genotype frequencies of TLR-4 gene polymorphisms were compared between chronic periodontitis and control groups. In this study, a total of 70 subjects who came to Department of Periodontics were selected. This comprises 30 healthy subjects and 40 patients who had chronic periodontitis. In the chronic periodontitis group, 23(57.5%) were males and 17(42.5%) were females whereas in the healthy group, 17(56.7%) were males and 13(43.3%) were females. The clinical parameters, mean age and mean of clinical attachment loss, were analyzed among the groups to determine their prediction for chronic periodontitis. The mean ages were 46 for patients and 37 for control. There was clinical attachment loss [CAL] at 7.5 mm in patients, whereas nill in controls.as shown in table 2.

Detection of PCR Products of TLR4 Genes and PCR-RFLP

The result of Thr399Ile TLR4 gene polymorphism was present in 406 bp fragment of Gel electrophoresis of PCR product for the Thr399Ile TLR4 gene as shown in figure 1.Followed by Restriction fragment length polymorphism (RFLP) analysis. The PCR products of Thr399Ile TLR4 gene polymorphism were digested with the enzyme HinfI that recognizes the sequence GANTC [7], and accordingly, it cuts PCR product of homozygous mutant genotype (TT) into two bands (377 and 29 bp), while heterozygous genotype (CT) is cut into three bands (406, 377, and 29 bp), Unfortunately, the smallest band (29 bp) was not visible on the gel because its small size, homozygous wild genotype is not affected (the band size is 406 bp) figure 2. There were three genotypes for this SNP among CP patients; CC, CT and TT with frequency of 77.5%, 10% and 12.5 % respectively, whereas, there were only two genotypes among control group; CC and TT respectively as shown in table 3. With frequency of 63.4% and 36.6% respectively with significance differences between patients and control (OR=3.807, 95%CI). However, the frequency of allele C (dominant) was higher among CP group (82.5%) than control (63.3) with significant difference (p=0.05).Similarly, there was significant difference in allele frequency between patients and control as the frequency of the dominant allele (C) among patients was 82.5% compared to 63.3% among control (OR= 2.829, 95%CI).

Thus the results for TLR-4 Thr399Ile SNP have shown that C allele frequency was 82.5 % in the CP groups and 63.3% in the healthy groups whereas T allele frequency was 17.5% in the chronic periodontitis group and 36.7 % in the healthy group as shown in table 4. For CT genotype (heterozygous mutant) frequency was found to be 10% in the CP group and zero in the healthy group whereas TT genotype (homozygous mutant) frequency was found 12.5 % in the CP group and 36.6% in the healthy group and CC genotype frequency was found 77.5% in the CP group and 63.4% in the healthy group .

Whereas the result of Asp299Gly TLR4 gene polymorphism was present in 249 bp fragment of Gel electrophoresis of PCR product for the Asp299Gly TLR4 gene as shown in figure 3. NcoI enzyme identifies the sequence CCATGG [7], whenever it presents in the nucleic acid and cuts exactly between the two Cs'. For homozygous wild type (AA), the enzyme does not work, and there will be a single band with 249 bp. For homozygous mutant genotype (GG), the enzyme cuts the two alleles, and the PCR product will appear as double bands of 223 and 26 bp; whereas the heterozygous genotype (AG) will appear as 3 bands of 249, 223, and 26 bp figure 4.

Unfortunately, the smallest band (26 bp) was not visible on the gel because its small size. There were three genotypes for this SNP among CP patients; AA, AG and GG with frequency of 67.5%,12.5% and 20% respectively, whereas, there were only two genotypes among control group; AA and GG respectively with frequency of 66.6 and 34.4% respectively with no significance differences between patients and control (OR=2.579, 95%CI= 0.745-19.47) as shown in table 4. However, the frequency of allele A (dominant) was higher among CP group (73.75) than control (66.6%) with significant difference ($p \leq 0.05$).

The results for TLR-4 Asp299Gly have shown that A allele frequency was (73.75 %) in the CP group and (66.6%) in the healthy group whereas G allele frequency was (26.25%) in the CP group and (33.4%) in the healthy group as shown in table 5. For AG genotype (heterozygous mutant) frequency was found to be (7.5%) in the chronic periodontitis group and zero in the healthy group whereas GG genotype (homozygous mutant) frequency was found (20%) in the CP group and (33.4%) in the healthy group and AA genotype frequency was found (67.5%) in the CP group and (66.6%) in the healthy group.

Thus, the results of this study have clearly shown that there is significant association between Thr399Ile in chronic periodontitis and control and whereas no association between Asp 299Gly in chronic periodontitis and control as far as Babylon population is concerned.

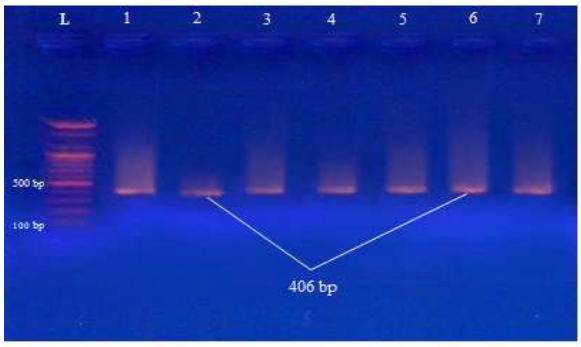

Figure 1: Gel Electrophoresis for TLR4 Thr399Ile PCR Products Visualized Under U. V Light After Staining with Ethidium Bromide. L: 1500 Bp Ladder; Lane 1-4: From Blood of CP Patients. Lane 5-7: From Blood of Controls. The Size of Product is 406 Bp

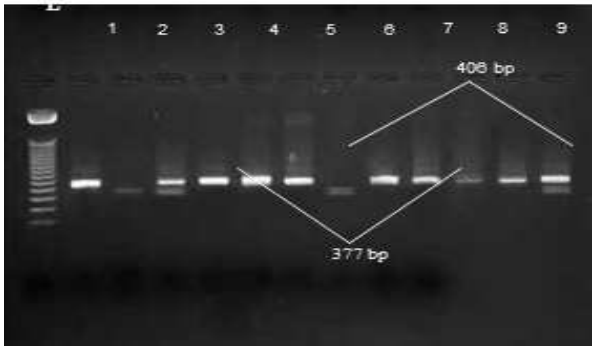

Figure 2: The 2% Agarose Gel Electrophoresis Showing the Restriction Digestion Patterns of Thr399Ile Polymorphisms of TLR4 Gene using Hinf I Enzyme. L: DNA Ladder. Lanes 1, 4, 9: Heterozygous Genotype (CT). Lane 2, 3, 5, 6, 7, 11, 12: Homozygous Wild Type (CC). Lanes 8, 10: Homozygous Mutant Type (TT)

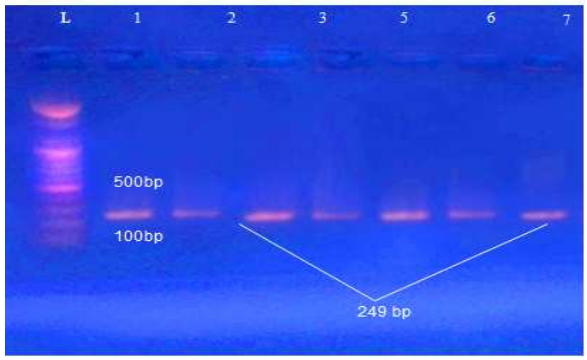

Figure 3: Gel Electrophoresis for TLR4 Asp299Gly PCR Products Visualized under U. V Light after Staining with Ethidium Bromide. L: 1500 Bp; Lane 1-5: From Blood of CP Patients. Lane 6-8: From Blood of Controls. The Size of Product is 249 Bp

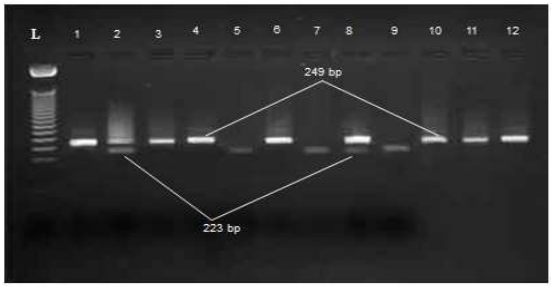

Figure 4: The 2% Agarose Gel Electrophoresis Showing the Restriction Digestion Patterns of Asp299Gly Polymorphisms of TLR4 Gene using Nco I Enzyme. L: 1500 DNA Ladder. Lanes 1, 2, 4,6, 9, 11, 12: Homozygous Wild Type (AA). Lane 3, 7: Heterozygous Genotype (AG). Lanes 5, 8, 10: Homozygous Mutant Genotype (GG)

Table 1: Specific Polymerase Chain Reaction Primers and Restriction Enzymes for the Two Single Nucleotide Polymorphism

Gene	Primer sequence (5'-3')	Amplicon size(bp)	Enzymes	Reference
TLR-4 Asp299Gly	F:GATTACATACTTAGACTACTACCTCGA R:GATCAACTTCTGAAAAAGCATTCLACC	249	*Nco* I	Hang et al.,2004
TLR-4 Thr399Ile	F:ATTGCTGTTCTCAAAGTGATTTTGGGACCAA R: CCTG AAGACT GGAGAGTGAGTTAAATGCT	406	*Hinf*I	

Table 2: Characteristics of the Study Groups

Clinical characters	Chronic periodontitis group			Healthy group		
	Male (%)	Female (%)	Total (%)	Male (%)	Female (%)	Total (%)
No. of Cases	23(57.5)	17(42.5)	40	17(56.7)	13(43.3)	30
Mean age	48	44	46.6	38	35	37
Mean CA loss	7.5			Nil		

Table 3: Genotype Distributions of the Thr399Ile Polymorphism in the TLR-4 Gene of Chronic Periodontitis and Healthy Groups

Groups	Genotype frequency		
CPG	CC (n, %)	CT (n, %)	TT (n, %)
Male	18	2	3
Female	13	2	2
Total	31(77.5)	4(10)	5(12.5)
Healthy	CC(n, %)	CT (n, %)	TT (n, %)
Male	11	0	6
Female	8	0	5
Total	19(63.4)	0	11(36.6)
P-value	0.006**	0.04*	0.094
OR(95%CI)	3.807	-	0.377

*p value < 0.05 is significant.

Table 4: Allele Frequencies of the Thr399Ile Polymorphism in the TLR-4 Gene of Chronic Periodontitis and Healthy Groups

Groups	Allele frequency	
CPG	C	T
	82.5%	17.5%
Healthy	C	T
	(63.3)	(36.7)
P-value	0.045*	
OR(95% CI)	2.829	

Table 5: Genotype Distributions of the Asp299Gly Polymorphism in the TLR-4 Gene of Chronic Periodontitis and Healthy Groups

Groups	Genotype frequency		
CPG	AA (n, %)	AG (n, %)	GG (n, %)
Male	16	3	4
Female	11	2	4
Total	27(67.5)	5(12.5)	8(20)
Healthy	AA(n, %)	AG(n, %)	GG(n, %)
Male	11	0	6
Female	9	0	4
Total	20(66.6)	0	10(33.4)
P-value	0.041*	0.021*	0.592
OR(95% CI)	2.579	-	0.750

Table 6: Allele Frequencies of the Asp299Gly Polymorphism in the TLR-4 Gene of Chronic Periodontitis and Healthy Groups

Groups	Allele frequency	
CPG	A	G
	73.75	26.25
Healthy	A	G
	(66.6)	(33.4)
P-value	0.459	
OR(95% CI)	1.444	

*** Alleles frequency by fisher exact test**

DISCUSSIONS

Gene polymorphisms that modify host response to the microbial challenge have been associated with different clinical forms of periodontitis [20].In case of TLR-4 Thr399Ile since CT heterozygous genotype is low percent only 10%, the source of CC genotype may be from GC and CC genotype is more than TT. So the CC is the wild type. And also for TLR-4 Asp 299Gly since AG heterozygous genotype is low percent only (12.5), the source of AA genotype may be from

AT and AA is more than GG. And also the AA is the wild type.

Several studies by Takashiba and Naruishi,[20] and Loos et al. [14] have shown that allelic variation in genes encoding molecules of the host defense system such as toll like receptors could affect the susceptibility and progression of periodontitis. Because TLR-4 offered a critical link between factors produced by pathogens and initiation of host defense, TLR-4 gene polymorphism may be important factors in susceptibility to bacterial infection and there by influence the inflammatory process .

Two common gene polymorphisms in the TLR-4 gene are Asp299Gly and Thr399Ile polymorphisms which have been shown to be associated with functional variations that prompt people to be less responsive to LPS, attenuate the TLR signaling pathway and leading to a blunted inflammatory response and thus have an increased risk of susceptibility to pathogenic bacterial infections [4].

Several studies have investigated the role of TLR-4 gene polymorphism in patients with periodontitis. In these, studies by Schröder et al. [19] in German population, Brett et al. [6] in Great Britain and by Fukusaki et al. [10] in Japanese population established association between chronic periodontitis and TLR-4 gene polymorphism. As frequency of polymorphisms and gene environment interactions differ among populations, the risk of a particular gene variant for a given genetic trait might be different in different population.

Recent study by Reddy et al. [17] was to evaluate association between the TLR-4 Asp299Gly and Thr399Ile gene polymorphisms and chronic periodontitis in sample of South Indian population. Thr399Ile alleles were found in 4% of CP patients and in 1% of periodontally healthy subjects. The prevalence of a Thr399Ile heterozygote was found to be 5% in the chronic periodontitis group and 1.67% in the periodontally healthy group, respectively. The TLR-4 Asp299Gly gene polymorphism was not detected in either chronic periodontitis or periodontally healthy groups .

In this study for TLR-4 Thr399Ile the gene polymorphism showed that the distribution of allele C was more compared to allele T (mutant variety). The frequency of occurrence of allele T (mutant variety) in chronic periodontitis is 20% and that of the healthy group is 36.7%. The results of this study are comparable to studies by Folwaczny et al. [8] and Laine et al. [13] who also reported that T allele frequency was 24.5% and 25.%, respectively, in chronic periodontitis groups. In contrast, a study by Izakovicova et al. [12] in Czech population found that T allele frequency was 93% in chronic periodontitis groups and this higher frequencies of T allele compared with other population are considered to reflect some ethnic difference in the relative frequencies of the TLR-4genotype in Czech population.

Another important finding in this study is that the results for TLR-4 Asp299Gly gene polymorphisms show low polymorphic mutants. These results were in contrast with the study by Hang et al. [11] and Okayama et al. [16] who reported that Asp299Gly polymorphism in TLR-4 is not present in Japanese and Chinese population. Thus, TLR-4 Asp299Gly gene polymorphism seemed to be rather low in the local population.

The results compatible with study done by Schroder et al. [19] who also reported significant association between chronic periodontitis groups and TLR-4Thr399Ile and no association between chronic periodontitis groups and TLR-4Asp299Gly gene polymorphism conducted in German population ($P \leq 0.002$).

Another important finding in this study is that the results for TLR-4 Asp299Gly gene polymorphisms show low polymorphic mutants. These results were in contrast with the study by Hang et al. [11] and Okayama et al. [16] who reported that Asp299Gly polymorphism in TLR-4 is not present in Japanese and Chinese population. Thus, TLR-4

Asp299Gly gene polymorphism seemed to be rather low in the local population.

The biological mechanism behind the association between TLR-4 gene polymorphisms and periodontal disease lies in the hypothesis that gene polymorphism of TLR-4 has been shown to be associated with functional changes that incline people to be less responsive to LPS, with decreased NF-κB activity and proinflammatory cytokine production and leading to blunted inflammatory response, thus increasing the risk of susceptibility to pathogenic bacterial infections [4,2,18].

CONCLUSIONS

There was significant association between TLR-4 Thr399Ile polymorphism and chronic periodontitisand no significant difference in the TLR-4 Asp299Gly gene polymorphism between the chronic periodontitis and controls

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تحليل العلاقة بين التغاير الجيني ل TLR-4 (TLR-4 Polymorphisms) و امراض اللثة المزمن عند سكان بابل العراق

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في هذه الدراسة ايضا تم التحري عن التغاير الجيني (TLR-4 Polymorphisms) باستخدام تقنية البلمرة و القطع بالأنزيم (PCR-RFLP). وتبين من النتائج وجود ثلاث انماط جينية في موقع طفرة *Thr399Ile* هما: CC وCT وTT ، اذ بلغت نسبة انتشارها (12.5%,10%,77.5%) في مجموعة المرضى على التوالي بينما هناك فقط نمطين في مجموعة السيطرة TT,CC وبنسبة انتشار(63.4%, 36.6%) على التوالي ، فيما بلغت نسبة انتشار الاليل C السائد في المرضى 82.5 % بالمقارنة للسيطرة 63.3% مع وجود اختلاف معنوي. وفي المقابل سجل وجود ثلاث انماط جينية في موقع طفرة *Asp299Gly* هي : AA و AG و GG وبنسبة انتشار 67.5 % و 12.5% و 20% في مجموعة المرضى على التوالي بينما هناك فقط نمطين في مجموعة السيطرةGG, AA وبنسبة انتشار,66.6% (34.4%) على التوالي. حيث سجلت نسبة انتشار الاليل A السائد (73.75%) في مجموعة المرضى بالمقارنة بمجموعة السيطرة (66.6%) وبفرق معنوي.